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Original Paper

Generation of fluorescent flowers exhibiting strong fluorescence by combination of fluorescent protein from marine plankton and recent genetic tools in *Torenia fournieri* Lind.

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Abstract Florescent proteins have been popularly used for studying genes and proteins of interest in various experiments at a cellular level, such as the analysis of intracellular localization and protein–protein interaction. However, the strength of fluorescence was insufficient for macro level observations of tissues or of the whole plant, and the fluorescent flowers that have been generated so far needed high-sensitive imaging equipment for the observation. Here we generated fluorescent *Torenia* flowers by the combined use of a high-performance fluorescent protein and the latest protein expression technologies, leading to the production of fluorescent proteins that can be easily and clearly observed. A coding sequence of a yellowish green fluorescent protein from the marine plankton *Chiridius poppei* (*CpYGFP*) was fused to the optimized sequences of the heat shock protein terminator and the 5′-untranslated region of the alcohol dehydrogenase gene of *Arabidopsis* to gain massive accumulation of the fluorescent protein. Strong fluorescence of CpYGFP was apparent in every part of the transgenic plant under the simple combination of a blue LED for excitation and an orange colored transparent acrylic filter for emission, while faint autofluorescence remained in the wild-type plants. By evaluating the combination of excitation wavelengths (excitation and emission filters) we were able to eliminate this undesired fluorescence. The fluorescent flowers could be used for ornamental purposes as well as for the analysis of fluorescent transgenic plants spatiotemporally in a nondestructive manner.

Key words: Fluorescent flower, CpYGFP, ADH enhancer, HSP terminator, florescence, *Torenia fournieri*.

The green fluorescent protein (GFP) was first identified and purified from the luminescence jellyfish *Aequorea victoria* along with the photoprotein aequorin (Morin and Hastings 1971; Morise et al. 1974; Shimomura et al. 1962); it can be photoactivated by near ultraviolet or blue excitation light and emits green fluorescence (Patterson et al. 2011). Dr. Osamu Shimomura, Dr. Martin Chalfie, and Dr. Roger Y. Tsien won the Nobel Prize in Chemistry in 2008 for the discovery and development of the GFP (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/).

Nowadays, there are many kinds of fluorescent proteins (FPs) with various colors and fluorescence properties because of the differences in the original organisms or in amino acid sequences (for review see Lippincott-Schwartz and Patterson 2003; Patterson 2011; Shaner et al. 2004; Subach et al. 2009; Wiedenmann et al. 2004). FPs have been used as powerful tools for studying proteins of interest in various experiments at cellular and tissue levels; analysis of protein–protein interaction (He et al. 2005) using fluorescence resonance energy transfer (FRET, Jares-Erijman and Jovin 2003; for review see Wallrabe and Periasamy 2005) and intracellular localization using FP-fusion proteins (Gurskaya et al.

Abbreviations: ADH, alcohol dehydrogenase; CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; HSP, heat shock protein; LED, light emitting diode; NOS, nopaline synthase; UTR, untranslated region; YGFP, yellowish-green fluorescent protein.

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2006; Ogawa et al. 1995; for review see Giepmans et al. 2006). In addition, GFP is used as a selection marker for generation of transgenic plants in several plant species (Rakosy-Tican et al. 2007; Vain et al. 1998; Zhang et al. 2001). In addition to these studies, FPs are widely utilized in a variety of key methodologies in cell biology (for reviews see Deponte 2012, Giepmans et al. 2006, and Tsien 1998).

On the other hand, utilization of these FPs in plant experiments sometimes causes problems because plants exhibit autofluorescence at the excitation wavelengths for FPs (Patterson et al. 2011) derived from chlorophyll (for review see Porcar-Castell et al. 2014), flavonol, and/or flavone (Smith and Markham 1998; for review see Pollastri and Tattini 2011). Therefore, an appropriate use of excitation/emission filters to avoid undesirable fluorescence is required for a precise observation. Although these FPs are useful for studies in cell and tissue biology at the micro level, strength and duration of fluorescence has been insufficient for observation at the whole-plant level; studies on protein functions for the development of petal shapes and color patterns, particularly those on spatiotemporal observation of plant organs or whole plants with a nondestructive manner.

The yellowish green FP was isolated from a marine plankton *Chiridius poppei* (CpYGFP; Masuda et al. 2006). CpYGFP exhibits fluorescent properties with excitation and emission maximum at 509 and 517 nm, respectively, and can be observed under non-UV light. Although the amino acid sequences are considerably different between *Aequorea victoria* green fluorescent protein (AvGFP) and CpYGFP (15.5%), protein structures of these FPs are similar (Suto et al. 2009). CpYGFP is useful and effective for plant study, because the fluorescence activity is more stable within a wide pH range in higher plants (approximately pH 4.5–7.2; Katsumoto et al. 2007; Martinière et al. 2013; for review see Taylor et al. 2012) than that of AvGFP (Masuda et al. 2006). In addition, CpYGFP is tolerant to chemical conditions where most proteins become less active; 8 M urea, 6 M guanidine–HCl, 50% acetone, 50% ethanol, and 50% methanol.

Various genetic tools effective for massive accumulation of transgene proteins in genetically modified (GM) plants, such as transcriptional terminators and translational enhancers, have been reported. Recent studies by Nagaya et al. (2010) on transcriptional terminators revealed that a terminator of the *heat shock protein 18.2* (*HSP*) gene of *Arabidopsis* (HSP-T) was most effective for the secure transcriptional termination, leading to effective gene expression. Accumulation of mRNA with HSP-T increases more than two times compared with the common *nopaline synthase* (*NOS*) terminator (NOS-T), and it also properly functions in rice and tomato (Hirai et al. 2011; Nagaya et al. 2010). The latest improved version of 878-bp HSP-T (HSP-T878) containing a matrix attachment region (MAR) increased the mRNA levels 1.5- to 2-fold higher than the previous version of HSP-T (250 bp) in stable transgenic plants (Matsui et al. 2014). On the other hand, the translational enhancer sequence Ω from tobacco mosaic virus (Gallie et al. 1991; Sleat et al. 1987) has long been used as a convenient tool for transgene expression. Other efficient translational enhancers have recently been isolated from several plant species; 5′-untranslated region (UTR) of *alcohol dehydrogenase* (*ADH*) in tobacco, rice, and *Arabidopsis* (Satoh et al. 2004; Sugio et al. 2008). Translational efficiency of *ADH* 5′-UTR is similar to that of the Ω sequence and enhances reporter gene expression approximately 30- to 100-hold when compared with the control plasmid pBI221 (Satoh et al. 2004). In addition, *ADH* 5′-UTR is effective in *Torenia* and *Chrysanthemum* (Aida et al. 2008).

The aim of this study is to generate flowers with strong fluorescence, never produced before. Visualization of gene expression using FP at the whole-plant level would contribute to the study of floral organ development as well as the commercialization of fluorescent flower products. Moreover, fluorescent flowers would be a good tool to gain public acceptance of GM plants (for review see Ohtsubo 2011). Here we generated *Torenia* flowers with strong fluorescence using *CpYGFP* gene and the powerful genetic tools of optimized HSP-T and *ADH* 5′-UTR. The fluorescent flowers highly expressed the *CpYGFP* gene and successfully accumulated the fluorescent protein, and their fluorescence under excitation light was clearly visible without any high-sensitive imaging equipment. In addition, we developed an efficient observation condition by optimizing the combination of an LED wavelength and excitation/emission filters. The fluorescence of CpYGFP was stably maintained under desiccated conditions, with the dried flower keeping strong fluorescence at least over a year. The fluorescent flower would be suitable not only for commercial use but also for studying spatiotemporal gene expression at the macro level in a nondestructive manner.

Materials and methods

Plant materials

Torenia fournieri cultivar 'Crown White' was used as the wild-type *Torenia* for the experiments. Maintenance of the plants was described in the previous report (Yamaguchi et al. 2011).

Plasmid construction

For the generation of a binary vector containing the *AvGFP* gene (L29345), pRI101-AN (Takara Bio Inc., Shiga, Japan) was used as the backbone vector. A promoter region of the pBE2113 binary vector (Mitsuhara et al. 1996) containing the Ω sequence (Gallie et al. 1991; Sleat et al. 1987) was

replaced with a cauliflower mosaic virus (CaMV) 35S promoter of the pRI101-AN DNA by the restriction sites, *Hin*dIII and *Xba*I, to produce pRI101-El2-Ω. A coding region of *AvGFP* was PCR amplified using primers GFP SalF (5′-aaagtcgacatggtgagcaagggcgagg-3′; *Sal*I site underlined) and GFP BamR (5′-tttggatcccttgtacagctcgtccatgc-3′; *Bam*HI site underlined) using pBI-sense, antisense-GW (Inplanta Innovations Inc., Kanagawa, Japan) as the template. The resulting fragments were digested with *Sal*I and *Bam*HI, and inserted into the same sites of pRI101-EL2-Ω to generate the pRI101-EL2-Ω:*AvGFP* binary vector.

For the generation of plasmid constructs containing the *CpYGFP* gene (AB185173), a DNA fragment containing the *CpYGFP* coding region was PCR-amplified using primers YGFP-A (5′-aaggcctacaaccttcaaaatcgagtcccg-3′; *Stu*I site underlined) and YGFP-B (5′-agagctcctacatgtctcttggggcgctgt-3′; *Sac*I site underlined), and pET101-*CpYGFP* (Masuda et al. 2006) as the template. The resulting fragments were digested with *Stu*I and *Sac*I, and inserted into the *Stu*I–*Sac*I gap of At1g77120 (*AtADH*)_5′UTR:*GUS* (Matsuura et al. 2013). Finally, the *Hin*dIII–*Eco*RI fragment was excised from this plasmid and cloned into the binary vector pRI909 (Takara Bio Inc.) to generate 1×*CpYGFP*. In the corresponding CpYGFP, three amino acids (Leu, Arg, Pro) after the start methionine were inserted to introduce a *Stu*I site.

A DNA fragment containing the *CpYGFP* coding region was PCR amplified from pET101-*CpYGFP* using primers YGFP-N (5′-aaatgcatgacaaccttcaaaatcgagtcc-3′; *Nsi*I site underlined) and YGFP-B. A DNA fragment containing CaMV 35S promoter and *AtADH* 5′-UTR was PCR amplified from At1g77120 (*AtADH*)_5′UTR:*GUS* using primers 35S XbaI-F (5′-aaatctagattagccttttcaatttcag-3′; *Xba*I site underlined) and ADH-N (5′-ttatgcattatcaacagtgaagaacttgcttttg-3′; *Nsi*I site underlined). These two fragments were ligated via their *Nsi*I sites and inserted into the *Xba*I–*Sac*I gaps of 1×*CpYGFP*. The resulting plasmid was digested with *Nsi*I, followed by treatment with T4 DNA polymerase to fix the *Nsi*I site and generate *AtADH*_5′UTR:*CpYGFP*:HSP-T. Furthermore, a *Sac*I–*Eco*RI HSP-T878 terminator fragment was excised from *AtADH-Fluc*-HSPT878 (Matsui et al. 2014) and inserted into the *Sac*I–*Eco*RI gap of *AtADH*_5′UTR:*CpYGFP*:HSP-T to generate *AtADH*_5′UTR:*CpYGFP*:HSP-T878. An *Xba*I–*Eco*RI fragment was excised from *AtADH*_5′UTR:*CpYGFP*:HSP-T878 and inserted into the *Spe*I–*Eco*RI gap of *AtADH*_5′UTR:*CpYGFP*:HSP-T878 to generate 2×*CpYGFP*. Furthermore, 3×*CpYGFP* was generated in the same manner.

Generation of transgenic Torenias

The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. After transformation of leaf discs by the *Agrobacterium*, the transgenic *Torenia* plants were screened and regenerated according to the previous reports (Aida 2008; Aida and Shibata 1995). Expression of introduced transgenes was confirmed by RT-PCR (Supplemental Figure S1).

Preparation of protoplasts

Protoplast preparation was performed according to the previous report in *Arabidopsis* (Contento et al. 2005). Petal and root protoplasts were prepared from *Torenia* by digesting cell walls overnight with the enzymatic solution at room temperature. The protoplasts in the enzymatic solution were directly observed using a digital microscope (VH-8000C; Keyence Co., Osaka, Japan) without straining by a nylon mesh filter.

Microscopic analysis

Torenia petals and petal protoplasts were observed using a digital microscope (VH-8000C) without fixation.

Preparation of dried flowers

To prepare dried flowers of wild-type and transgenic *Torenia*, excised plant materials were immediately embedded in dried silica gels (Wakogel C-300; Wako, Osaka, Japan) and desiccated for 2 months.

Evaluation of fluorescence strength

Fluorescence strength was visually evaluated by comparing the photographs taken under the same imaging condition (including LED light volume, f-ratio, ISO speed, shutter speed, and focal length) in each experiment of Figures 2, 5, and 6. Each condition is shown in the figure legends. Photographs were taken under LED lights (model VBL-SL150 equipped with three different LEDs of peak wavelengths 459 nm, 474 nm, and 500 nm as in Supplemental Figure S2; Valore Corp., Kyoto, Japan) with a Canon EOS kiss Digital N camera and a Canon EF-S 18–55-mm lens (Canon, Tokyo, Japan), in combination with excitation and/or emission filters (Supplemental Figure S3, S6: OBF; Mitsubishi Rayon Co., Ltd. Tokyo, Japan, YBF; Nitto Jushi Kogyo Co., Ltd. Kogyo, Tokyo, Japan, HPF_{510}, $BPF_{350–490}$ and $BPF_{465–475}$; Asahi Spectra Co., Ltd. Tokyo, Japan). Because the main purpose of this study was to obtain fluorescent flowers suitable for visual observation-based experiments or display, visual evaluation with photographs was selected as the most simple and effective method.

Results

Generation of fluorescent Torenia flowers

To generate fluorescent *Torenia* flowers, we first attempted a combination of commercially available *AvGFP* genes and a binary vector containing the CaMV 35S promoter (Figure 1, upper). Expression of introduced genes in the transgenic plants was examined by RT-PCR (Supplemental Figure S1). In Figure 2, a blue LED excitation light with a peak wavelength of 459 nm (LED_{459}; Supplemental Figure S2) and an orange colored transparent acrylic plate as an emission filter (Supplemental Figure S3, upper) were used for fluorescence observation. Fluorescence was hardly observed in any of the five *AvGFP* transgenic lines

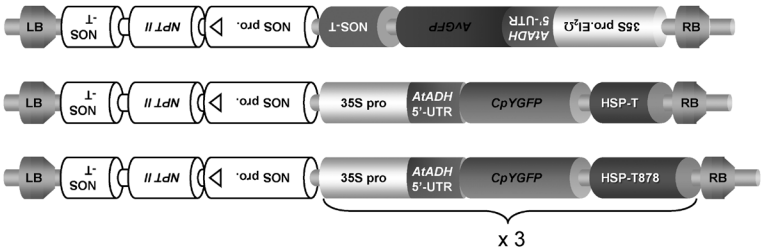

Figure 1. Plasmids used for the production of fluorescent transgenic *Torenia* plants. 35S pro; 35S promoter from cauliflower mosaic virus (CaMV), AtADH; *Arabidopsis alcohol dehydrogenase*, NOS-T; NOS terminator, NPTII; *neomycin phosphotransferase* gene for kanamycin resistance, HSP-T; terminator of heat shock protein (HSP), HSP-T878; terminator of 878-bp HSP, RB; right border, LB; left border, CpYGFP; *Chiridius poppei* yellowish green fluorescent protein, AvGFP; *Aequorea victoria* GFP.

(Figure 2C), while accumulation of *AvGFP* transcripts was confirmed in four of the five lines (Figure S1). There are two possible explanations for this result: instability of the AvGFP activity and shortage in AvGFP accumulation. To eliminate these possibilities, we examined another fluorescent protein CpYGFP and a transcriptional terminator sequence HSP-T to increase protein stability and transcript accumulation (Figure 1, middle). Fluorescence activity of CpYGFP is more stable than AvGFP under a lower pH condition (Masuda et al. 2006), which is close to the environment inside the plant cells. HSP-T increases transcriptional efficiency by stabilizing the 3′-end formation of pre-mRNA; thus, upregulating introduced gene expression two-fold more than NOS-T (Nagaya et al. 2010). In addition, unnecessary cloning sequence between *AtADH* 5′-UTR (Sugio et al. 2008) and the *CpYGFP* coding region was removed (Supplemental Figure S4) because direct connection of *AtADH* 5′-UTR to the first methionine of the following coding sequence enhances translational efficiency (Satoh et al. 2004). A considerable number of *Torenia* plants transformed with this improved gene construct (1×*CpYGFP*) clearly exhibited strong fluorescence observable without using any image-enhancing equipment (Figure 2D, 1×*CpYGFP* plants). To gain stronger fluorescence, HSP-T (250 bp) in 1×*CpYGFP* was replaced with its longer version HSP-T878 (Matsui et al. 2014), and the expression cassette was tandemly triplicated (3×*CpYGFP*; Figure 1, lower). A significant number of *Torenia* plants transformed with this improved gene construct exhibited an excellent strong fluorescence never observed before (Figure 2E, 3×*CpYGFP* plants).

Observation of fluorescence in the Torenia plants

To examine the stability and amount of CpYGFPs accumulated in transgenic plants, crude extracts prepared from leaves and petals of 3×*CpYGFP* plants were electrophoretically separated, then protein accumulation and fluorescence strength of CpYGFP were evaluated. Four independent transgenic lines with strong fluorescence (line numbers 29, 34, 42, and 56) were selected for the experiment. In SDS-PAGE, putative protein bands for CpYGFP were found in the leaves and petals of the 3×*CpYGFP* plants (Figure 3A, arrowheads), but not in the wild-type control. These crude extracts were further examined by native PAGE, and the strong fluorescence of the corresponding protein bands were clearly observed (Figure 3B).

The strong CpYGFP fluorescence emerged from the early stage of the transformation. When the 3×*CpYGFP* construct was introduced into *Torenia* by *Agrobacterium*-mediated transformation (Aida and Shibata 1995), many of the putative transformed calli generated on the leaf disks exhibited strong fluorescence (Figure 4A, red arrowhead). Transgenic plants regenerated from these calli exhibited strong fluorescence in the whole plant body including stems and roots (Figure 4B, 4C). Because these transgenic plants have been maintained by vegetative propagation because of their heterozygosity (Yamaguchi et al. 2011), this fluorescence was maintained at least >18 months, from the time point of shoot formation until the writing of this manuscript.

To confirm intracellular localization of CpYGFP, fluorescence microscopic observation of the petals of 3×*CpYGFP* plants was performed. The fluorescence observed in the cross section of the petal was uniformly strong without revealing the intracellular localization (Figure 4D, upper). Therefore, we examined the protoplasts prepared from the petals of 3×*CpYGFP* plants (Figure 4D, lower; $n=30$). CpYGFP fluorescence was mainly localized in the cytoplasm. The AvGFP fluorescence, as a control, was also mainly observed in a cytosolic localization (Supplemental Figure S5; $n=9$) and no preferential localization to the nucleus and vacuole. No fluorescence was observed in the protoplasts prepared from wild-type plants under the same condition (Supplemental Figure S5; $n=21$).

These results suggest that the CpYGFP protein is stably accumulated and maintained in every tissue and organ of the plant body, and the strong fluorescent even in extracellular environment such as the electrophoretic condition. *CpYGFP* transgenic plants would be useful in various experimental situations because strong

fluorescence is easily observable not only at the cell and tissue levels but also at the organ and whole-plant levels.

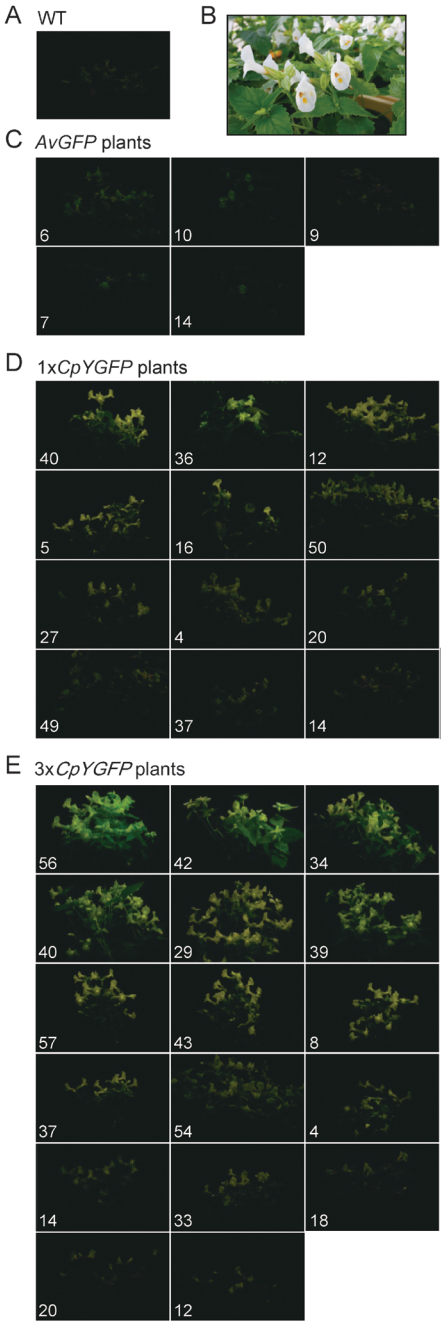

Figure 2. Comparison of florescence in transgenic *Torenia* plants. Fluorescence was observed in wild-type (WT) (A). WT plants ('Crown White') were photographed in a contained greenhouse (B). Fluorescence was observed in *AvGFP* plants (C), 1×*CpYGFP* plants (D), and 3×*CpYGFP* plants (E). Numbers in each box indicate the numbers of transgenic lines. To compare the strength of their fluorescence in transgenic *Torenias* and efficiency of each fluorescent construct, WT plants ('Crown White') and fluorescent transgenic plants were photographed under the same condition except for the WT plants that were photographed under the visible light (B). Fluorescence was observed with a blue LED_{459} and the orange barrier filter. Image acquisition condition: F5.6, ISO100, 0.5 s exposure, and focusing length 28 mm.

Comparison and improvement of observable CpYGFP fluorescence using various optical filter combinations

The simplest way to observe CpYGFP fluorescence in transgenic *Torenia* (Figure 5A; under visible light) is the use of a commercial blue LED light in combination with an orange colored transparent acrylic plate. However, unnecessary plant autofluorescence is also observed with such a simple combination. As observed in the wild-type plants in Figure 5B (upper left), red fluorescence in stems and leaves as well as faint green fluorescence in petals was detected. The former arose from chlorophyll *a*, which has its absorption maximum around 450 nm (for review see Porcar-Castell et al. 2014) and the latter from flavonols whose long-wavelength end of the excitation spectrum in petals reaches around 450 nm (Smith and Markham 1998). These autofluorescence

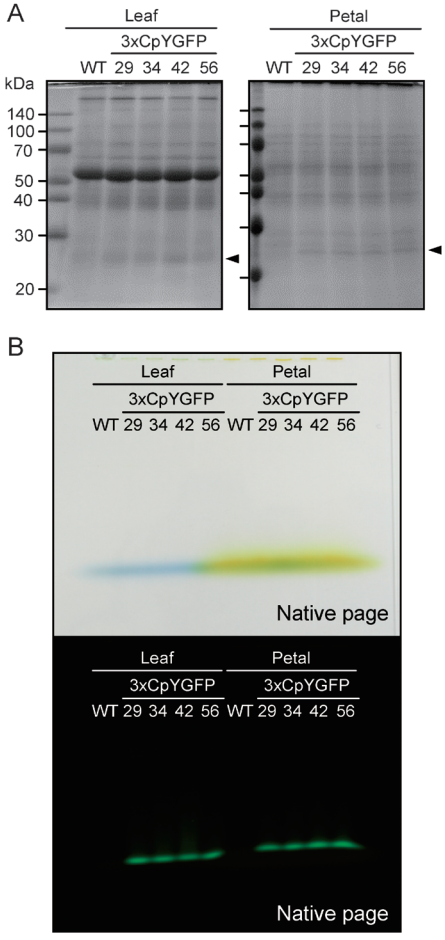

Figure 3. SDS-PAGE and native PAGE of crude extract from transgenic *Torenia* plants. (A) The crude extracts from leaves and petals of wild-type (WT) and 3×*CpYGFP* plants were subjected to SDS-PAGE on a 12% (w/v) polyacrylamide gel and stained with CBB. Arrowheads indicate putative CpYGFP proteins (24.7 kDa; Masuda et al. 2006). The numbers on the left side indicate the molecular weight (kDa). (B) The crude extracts were loaded on 10% polyacrylamide gels (native PAGE; upper). The electrophoresed CpYGFP protein was visualized with a blue LED_{459} and an emission filter (lower). Numbers on the polyacrylamide gels indicate the numbers of 3×*CpYGFP* transgenic lines.

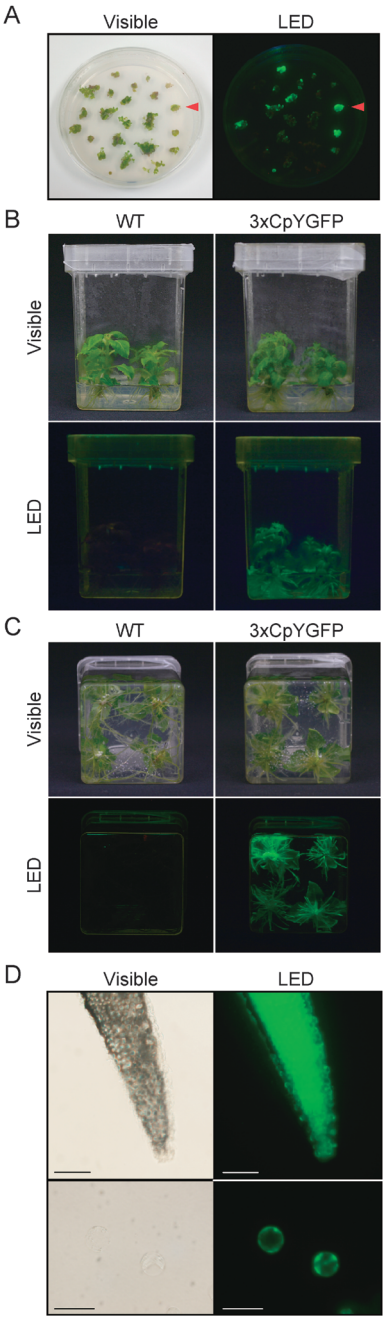

Figure 4. Observation of 3×*CpYGFP* plants under various conditions. (A) Fluorescence of CpYGFP was observed in calli, where the 3×*CpYGFP* construct was introduced (red arrowhead). (B) Observation of fluorescence in juvenile WT (left) and 3×*CpYGFP* plants (right), cultured in a plant box. (C) Observation of fluorescence in roots of WT (left) and 3×*CpYGFP* plants (right). (D) Intracellular localization of the CpYGFP protein in 3×*CpYGFP* plants was examined in petal sections (upper) cut by a razor and protoplasts (lower) from petals using a digital microscope. Scale bar=100 μm in upper panels and 50 μm in lower panels.

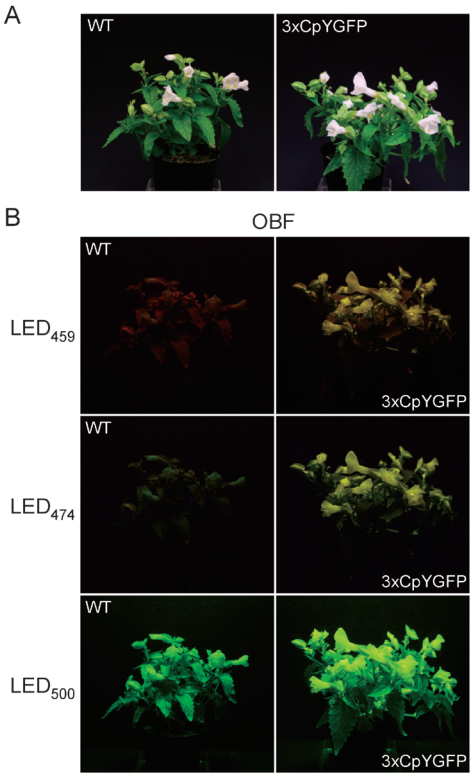

Figure 5. Difference of visibility in 3×*CpYGFP* plants using LEDs of different wavelength. (A) Photographs of WT and 3×*CpYGFP* plants under visible light. (B) Fluorescence of WT (left panels) and 3×*CpYGFP* plants (right panels) were compared under three types of LED wavelengths, 459 nm (LED_{459}; upper), 474 nm (LED_{474}; middle), and 500 nm (LED_{500}; lower). WT and 3×*CpYGFP* plants were photographed using an LED and an orange barrier filter. Image acquisition condition: F5.6, ISO100, 2.0-s exposure, and focusing length 28 mm. OBF; orange barrier filter.

patterns were assumed to be excited by the LED_{459} light. To eliminate this unnecessary background and improve the observation performance, other LEDs with different wavelength characteristics were tested. Taking maximum excitation (509 nm) and emission (517 nm) wavelength of CpYGFP into consideration, LEDs with peak wavelengths at 474 nm and 500 nm (LED_{474} and LED_{500} respectively in Supplemental Figure S2) were selected for comparison (Figure 5B). Under the LED_{474} excitation, chlorophyll *a*-derived red fluorescence fairly disappeared, while the flavonol-derived faint green fluorescence remained in wild-type plants (Figure 5B, middle). Under LED_{500} excitation, strong fluorescence was observed both in wild-type and 3×*CpYGFP* plants (Figure 5B, lower). This was because of excessive overlapping of the excitation light on the CpYGFP fluorescence. These results indicated that LED_{474} is effective for the reduction of chlorophyll autofluorescence during CpYGFP fluorescence observation, whereas complete elimination of plant autofluorescence requires further improvement of the observation method.

Because the excitation and emission wavelengths in CpYGFP are very close, optimization of excitation and emission filters are required for further reduction of autofluorescence and enhancement of optical contrast. Therefore, we tested the combinations of the three LED wavelengths, three high-pass emission filters

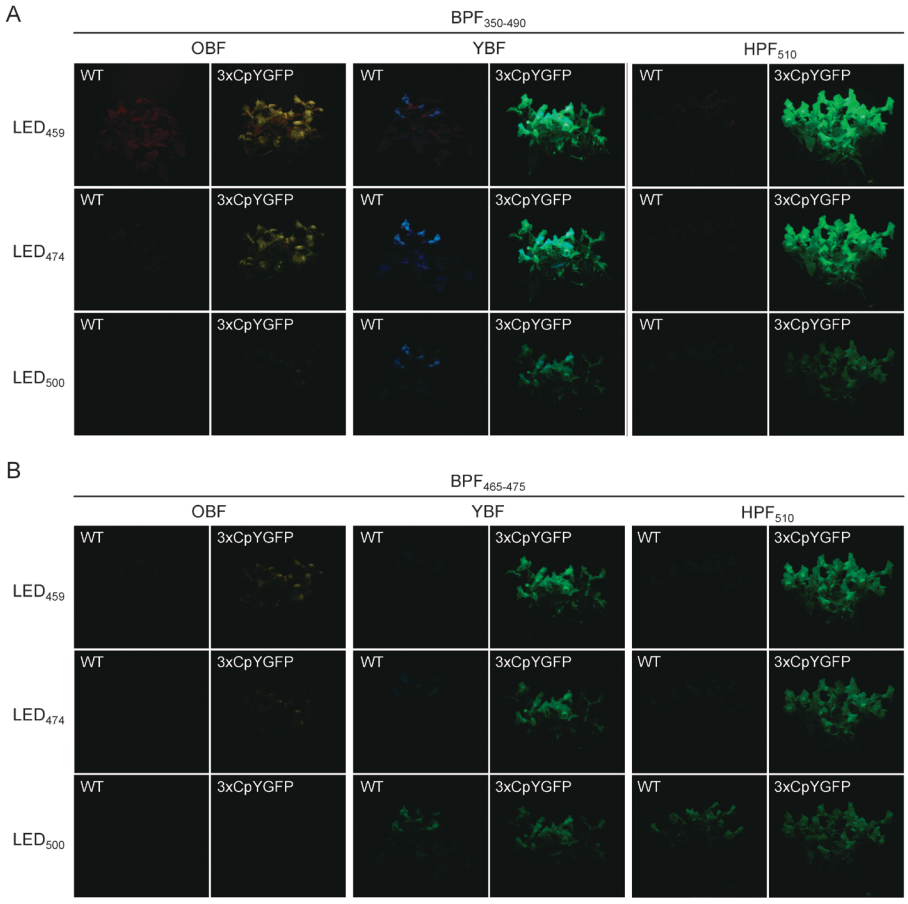

Figure 6. Fluorescence observation of 3×*CpYGFP* plants under various combinations of excitation lights and optical filters. The 3×*CpYGFP* plants were observed with combination of an excitation filter, a barrier filter, and an LED. (A) Fluorescence was observed in 3×*CpYGFP* plants with a $BPF_{350–490}$. (B) Fluorescence was observed in 3×*CpYGFP* plants with a $BPF_{465–475}$. Fluorescence was observed with a combination of three types of LEDs (LED_{459}, LED_{474}, and LED_{500}) and three types of barrier filters (OBF, YBF, and HPF_{510}) in addition to an excitation filter. Image acquisition condition: F5.6, ISO100, 2.5-s exposure, and focusing length 28 mm.

(Supplemental Figure S3), and two band-pass excitation filters (Supplemental Figure S6). The fluorescence of wild-type and 3×*CpYGFP* plants observed under each condition are photographically represented in Figure 6.

The yellow barrier filter (YBF) and the 510-nm high-pass filter (HPF_{510}) increased observable fluorescence from 3×*CpYGFP* plants. The orange barrier filter (OBF) considerably reduced CpYGFP fluorescence, probably by shadowing the short-wavelength part of the CpYGFP fluorescence. The 465–475-nm band-pass filter ($BPF_{465–475}$) and the 350–490-nm band-pass filter ($BPF_{350–490}$) were equally effective for reducing plant autofluorescence. However, the fluorescence strength was totally decreased in $BPF_{465–475}$ because of the lower excitation energy than $BPF_{350–490}$. The sharpest fluorescence contrast between wild-type and 3×*CpYGFP* plants was observed under the combination of LED_{459}/$BPF_{350–490}$/HPF_{510}, while HPF_{510} is not suitable for public display because of its optical property (as discussed later). Any combination using LED_{500} would cause leakage of excitation light, probably because of the proximity of the wavelength characteristics between LED

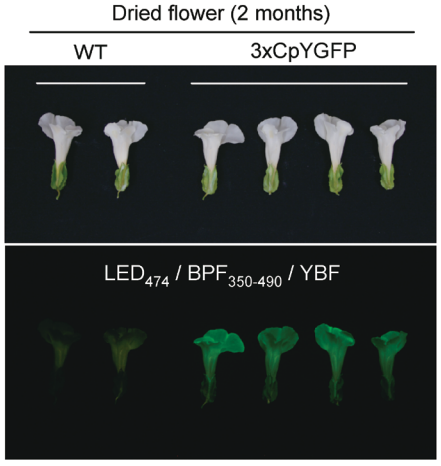

Figure 7. Dried flower produced with 3×*CpYGFP* plants. Two months after the wild-type and 3×*CpYGFP* flowers were dried with the fine-grained silica gels, the dried flowers were photographed. Dried flowers were observed under visible light (upper) and the combination of LED_{474}/$BPF_{350–490}$/YBF (lower).

and CpYGFP fluorescence. Therefore, we have succeeded in improving the observation performance of CpYGFP fluorescence by increasing detectable fluorescence strength and reducing plant autofluorescence, while the combination of an excitation wavelength and excitation/emission filters can be improved to avoid excitation light leakage.

Discussion

Fluorescence properties of 3×CpYGFP plants

By the combination of CpYGFP and high-efficiency expression vector, we have succeeded in producing *Torenia* plants exhibiting strong fluorescence. In addition, we have been able to improve the observable CpYGFP fluorescence of transgenic plants and eliminate plant autofluorescence by optimizing the combination of excitation light and excitation/emission filters, although the number of combinations we tested was not very high. The bright fluorescence was stably observed in every part of the plant body including the thin-layered tissues such as petals (Figure 4). In our experiments, continuous excitation of CpYGFP under natural daylight containing excitation light, as well as >10 h of continuous LED excitation did not affect fluorescence at all. No growth inhibition or morphological defect was observed in the transgenic plants highly expressing and accumulating CpYGFP. Therefore, CpYGFP could be suitable for prolonged observation.

CpYGFP expressed in the transgenic plants was localized in the cytoplasm (Figure 4D). Because the vacuole occupies a large part of the plant cell, it could be a good storage for such abundantly expressed proteins. Because CpYGFP is stable even at a low pH or in a degenerative chemical environment, the vacuolar sorting signal (for review see Vitale and Hinz 2005) may be a tool to enhance fluorescence strength. We are now testing this possibility, and successful sorting of CpYGFP to vacuole may provide a stronger fluorescence signal than 3×*CpYGFP* plants.

On the other hand, other combinations of excitation lights and excitation/emission filters for maximizing observable CpYGFP fluorescence can be attempted. Even under the most efficient combination of light and filters found in this study (Figure 6; $LED_{459}/BPF_{350–490}/HPF_{510}$), the excitation and emission energies are approximately cut off up to 50% in total; thus, only 50% of the potential fluorescence of *CpYGFP* plants is detectable under the condition. Intensive and strategic improvement, including modification of the excitation and fluorescence characteristics of CpYGFP by protein engineering, will enable us to detect even the faint action of genes and proteins without complex procedures.

Effective usage of highly stable fluorescent proteins in plants

CpYGFP maintained fluorescence even under severe conditions. CpYGFP fluorescence in transgenic plants remained observable even after complete desiccation of the plants by embedding in fine-grained silica gels (Figure 7; $LED_{474}/BPF_{350–490}$/YBF) and has been observable for >1 year (data not shown). In addition, this fluorescence remained detectable after embedding the plants in silicon resin (Supplemental Figure S7) while the fluorescence strength decreased under the present condition. This characteristic provides several advantages not only for the development of commercial products, such as dried flowers or resin-embedded specimens, but also for experimental conditions under various chemical environments. On the other hand, luciferase (for review see Jiang et al. 2008) could not be used for the same purpose because emission of fluorescence by luciferase requires the presence of water for enzyme activity.

Commercialization of transgenic fluorescent flowers requires the assessment of biodiversity impact according to the domestic laws or related regulations of The Cartagena Protocol on Biosafety in each country. With the objective of eliminating biodiversity impact, *Torenia* used in this study is a good candidate because there is no wild species crossed with *Torenia fournieri* at least in Japan (Miyazaki et al. 2007). Because *CpYGFP* expression construct can also function in other floricultural plants and white-flowered varieties, generation and commercialization of fluorescent flowers, such as chrysanthemums, roses, carnations and cyclamens, will be possible in the future; however, some of them require additional modifications with respect to their fertility to reduce impacts on biodiversity. White-flowered varieties should be preferred because some of the petal pigments significantly reduce CpYGFP fluorescence, probably by shading the excitation/emission light. For this reason, we failed to generate fluorescent flowers using purple colored *Torenia* cultivar 'Crown violet' (CV; Supplemental Figure S8). *Torenia* petals contain five anthocyanins with absorbance maximum from 514 nm to 526 nm (Sasaki et al. 2010); therefore, a part of the excitation light from LED_{459} and/or the CpYGFP fluorescence (emission maximum at 517 nm) may be absorbed by the pigments.

This strong and persistent nature of CpYGFP fluorescence may provide a new method for functional analyses of plant genes and proteins. For example, it will enable us to monitor spatiotemporal behaviors of very low expressed genes at the organ or whole-plant levels in a nondestructive manner without any valuable imaging equipment. In addition, it will enable efficient secondary excitation in FRET-based analyses.

Further development and improvement of fluorescent flowers

Our goal is to elucidate how colors, patterns, and shapes are regulated in ornamental flowers and discover their applications in modifying floral traits. Usage of floral organ-specific promoters, such as floral MADS-box gene promoters (Sasaki et al. 2010; Sasaki et al. 2011) or pigment biosynthesis genes in combination with the *CpYGFP* construct, may be effective for this purpose. Nondestructive and continuous monitoring of gene expressions during floral development will provide a new spatiotemporal perspective on the regulation of floral traits. Generation of transgenic plants for this purpose is under investigation.

On the other hand, usage of fluorescent flowers for commercial purposes requires some different approaches. For example, the most efficient combination of excitation light and filters found in this study (Figure 6; LED_{459}/$BPF_{350–500}$/HPF_{510}) is not suitable for ornamental purposes for the following reasons: the special optical characteristic of HPF_{510} and the cost. Because HPF_{510} is a reflective filter, severe leakage of the excitation light occurs if out of the viewing angle (Supplemental Figure S9B, lower). In addition, HPF_{510} is 10 times more expensive than YBF; therefore, it is unsuitable for exhibition or commercial products usage.

Because the emission filters used in this study have their own colors, a proper selection for eliminating a wrong visual perception under normal lights would also be necessary. Remarkably, the comfortable combinations of the light and filters for exhibition were LED_{459}/$BPF_{350–490}$/YBF, LED_{474}/$BPF_{350–490}$/YBF, and LED_{459}/$BPF_{465–475}$/YBF (Figure 6). High-contrast observation with solid colored fluorescence is not necessarily desirable in terms of visual impact of fluorescent flowers because the solid color significantly ruins the stereognostic sense and the invisible control; thus, making us feel as if we are viewing a normal plant under green light. Flexible evaluation strategies based on the practical use of transgenic plants would be necessary for further production and improvement of fluorescent flowers.

Fluorescent flowers have high potentials for cultural and educational purposes by showing the fun of science; thus, contributing to gaining public acceptance of GM plants through the use of fresh flowers, dried flowers, and embedded specimens.

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Materials and Methods for Supporting Analyses

Plant materials

Torenia fournieri cultivars ‘Crown White’ (CW) and ‘Crown Violet’ (CV) was used for the experiments. Maintenance of the plants was described in the previous report (Yamaguchi et al. 2011).

Expression analysis by RT-PCR

Total RNA was isolated from leaves by TRIzol (Invitrogen) and cDNA was synthesized from the total RNA using a cDNA synthesis kit (TOYOBO, Osaka, Japan). RT-PCR was performed with KOD Plus 2 (TOYOBO) using gene-specific primer sets for *AvGFP* (forward; 5′-AAAgtcgacATGGTGAGCAAGGGCGAGG-3′, and reverse; 5′-TTTggatccCTTGTACAGCTCGTCCATGC-3′), *CpYGFP* (forward; 5′-ATGACAACCTTCAAAATCGAGTCC-3′, and reverse; 5′-CTACATGTCTCTTGGGGCGCTG-3′), and *TfACT3* (forward; 5′-AAATACAGTGTTTGGATCGGAGGTTC-3′, and reverse; 5′-GAATAGCACACAGAGAATAGCAAACC-3′). The quality and quantity of each cDNA sample was examined by the transcript levels of the *TfACT3* gene (AB330989), used as an internal control.

Supplemental Figure Legends

Fig. S1. Confirmation of expression of fluorescence genes by RT-PCR. (A) Expression of *AvGFP* gene was examined in *AvGFP* transgenic plants. (B) Expression of *CpYGFP* was examined in 1×*CpYGFP* plants. (C) Expression of *CpYGFP* was examined in 3×*CpYGFP* plants. The *TfACT3* gene was used as an internal control. PCR cycles are indicated on the right of each column.

Fig. S2. Emission spectra of LEDs used as excitation lights. (A) Emission spectrum of a LED_{459} (VBL-SD150-B_Spectol), whose emission maximum wavelength is 459 nm. (B) Emission spectrum of a LED_{474} (VBL-SL150-B_Spectol), whose emission maximum wavelength is 474 nm. (C) Emission spectrum of LED_{500} (VBL-SL150-BB (500)), whose emission maximum wavelength is 500 nm. The information of the emission spectrum data was provided from the manufacturer.

Fig. S3. Transmission spectra of barrier filters. (A) Transmission spectrum of an orange barrier filter (OBF). (B) Transmission spectrum of a yellow barrier filter (YBF). (C) Transmission spectrum of 510-nm high-pass filter (HPF_{510}; LV0510). The information of transmission spectrum data was provided from the manufacture.

Fig. S4. Schematic representation of the improvement of fluorescence constructs. To gain a better translational efficiency, extra sequences between *AtADH* 5′-UTR and coding region of *AvGFP* (upper) were removed for the *CpYGFP* construct (lower). Red arrows indicate the translational initiation site (first methionine) of fluorescence genes.

Fig. S5. Observation of fluorescence in protoplast of *AvGFP* transgenic *Torenia* plants and wild-type *Torenias*. (A) Protoplasts in the roots of *AvGFP* transgenic plants were observed with a digital microscope under visible light (left) and a blue light (right). A representative image is shown in this figure (number = 9). (B) Protoplasts in petals of wild-type *Torenias* were also observed with a digital microscope under visible light (left) and a blue light (right). A representative image is shown in this figure (number = 21). Scale bar = 50 μm.

Fig. S6. Transmission spectra of excitation filters. (A) Transmission spectrum of a 350–490-nm band-pass filter ($BPF_{350–490}$; SV0490). (B) Transmission spectrum of a 465–475-nm band-pass filter ($BPF_{465–475}$; MZ0470 M.C.470/10 nm). The information of transmission spectrum data was provided from the manufacturer.

Fig. S7. A prototype of resin-embedded *Torenia* specimens. Fluorescence was observed in the resin-embedded specimen using the 3×*CpYGFP* plants (A), but the plants in the resin was partially translucent (B).

Fig. S8. Nonfluorescence in flowers of *CpYGFP*-transformed *Torenia fournieri* 'Crown Violet.' (A) Photographs of wild-type *Torenia* 'Crown white' (CW) used in this study, 'Crown violet' (CV), 3×*CpYGFP* transgenic line-42 (3×*CpYGFP*-42) generated in CW, and 1×*CpYGFP* transgenic line-4 (*CpYGFP*-4) generated in CV under the visible light. (B) Fluorescence was observed in CW, CV, 3×*CpYGFP*-42 in CW, and *CpYGFP*-4 in CV under the combination of LED_{474}, YBF, and $BPF_{465-475}$. Red arrowheads indicate flowers of *CpYGFP*-4 in CV. (C) Expression of *CpYGFP* was examined by RT-PCR in

CpYGFP plants generated using CV. The *TfACT3* gene was used as an internal control. PCR cycles are indicated on the right of each column.

Fig. S9. Observation of fluorescence in transgenic flowers using HPF_{510} as a barrier filter. (A) Photographs of WT (left) and 3×*CpYGFP* plants (right) under visible light. (B) Observation of fluorescence at a right angle (upper) and from an oblique angle (under) in WT (left) and 3×*CpYGFP* plants (right) using the combination of LED_{459}, HPF_{510}, and $BPF_{465–475}$. Red arrowheads indicate a blue light which transmitted through HPF_{510}.

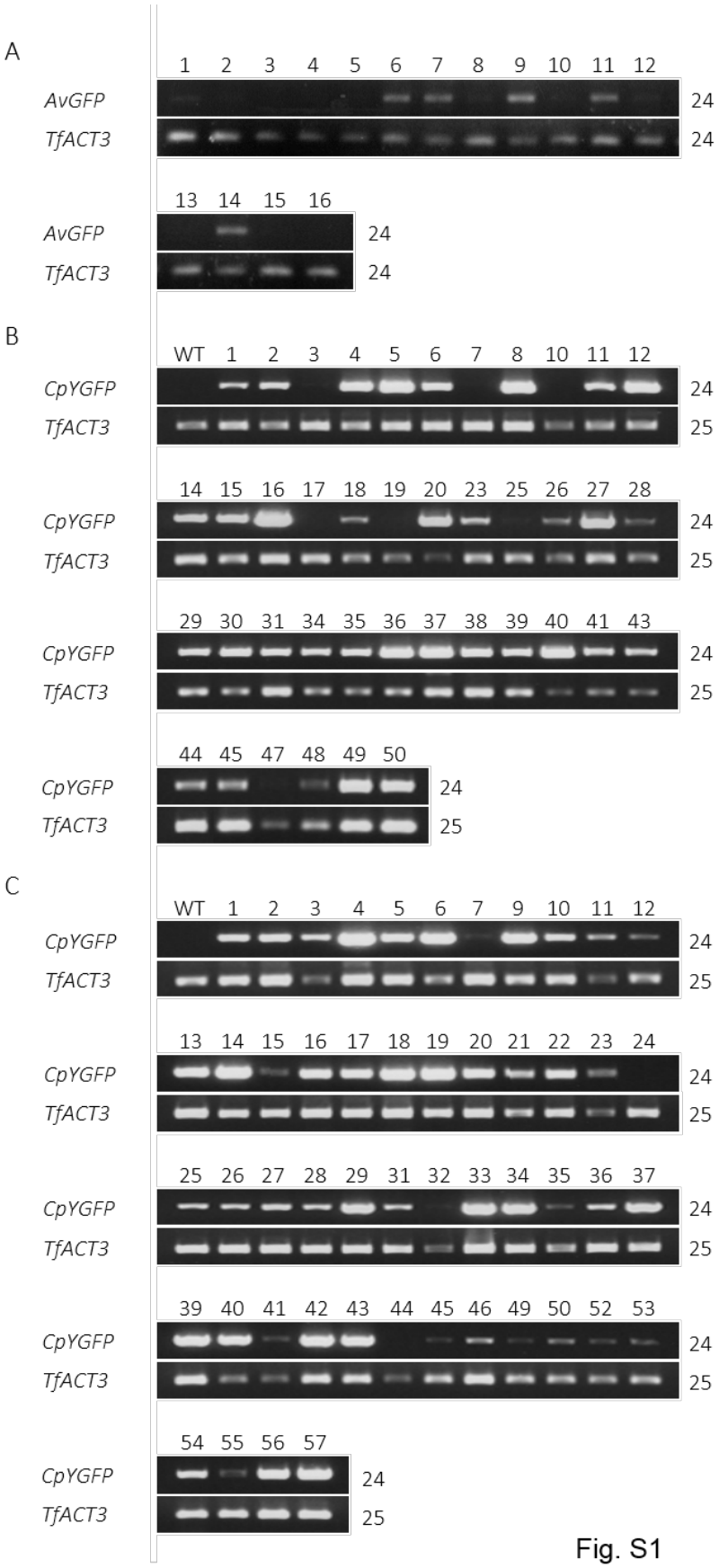

A
1 2 3 4 5 6 7 8 9 10 11 12
AvGFP
TfACT3
24
24
13 14 15 16
AvGFP
TfACT3
24
24
B
WT 1 2 3 4 5 6 7 8 10 11 12
CpYGFP
TfACT3
24
25
14 15 16 17 18 19 20 23 25 26 27 28
CpYGFP
TfACT3
24
25
29 30 31 34 35 36 37 38 39 40 41 43
CpYGFP
TfACT3
24
25
44 45 47 48 49 50
CpYGFP
TfACT3
24
25
C
WT 1 2 3 4 5 6 7 9 10 11 12
CpYGFP
TfACT3
24
25
13 14 15 16 17 18 19 20 21 22 23 24
CpYGFP
TfACT3
24
25
25 26 27 28 29 31 32 33 34 35 36 37
CpYGFP
TfACT3
24
25
39 40 41 42 43 44 45 46 49 50 52 53
CpYGFP
TfACT3
24
25
54 55 56 57
CpYGFP
TfACT3
24
25

Fig. S1

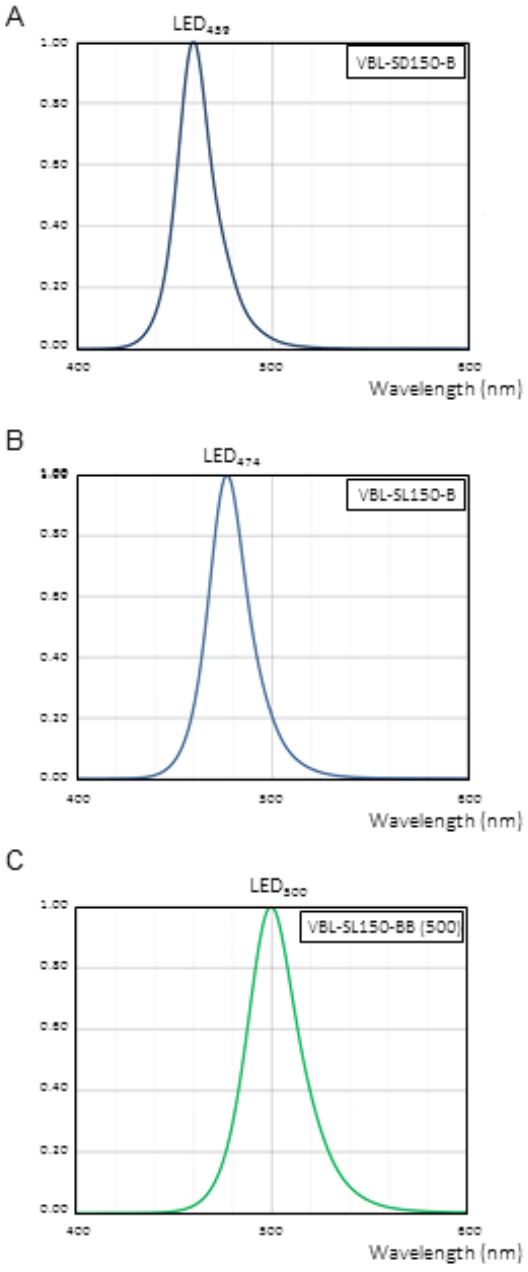

A
LED459
VBL-5D150-B
1.00
0.80
0.60
0.40
0.20
0.00
400
500
600
Wavelength (nm)
B
LED474
VBL-5L150-B
1.00
0.80
0.60
0.40
0.20
0.00
400
500
600
Wavelength (nm)
C
LED500
VBL-5L150-BB (500)
1.00
0.80
0.60
0.40
0.20
0.00
400
500
600
Wavelength (nm)

Fig. S2

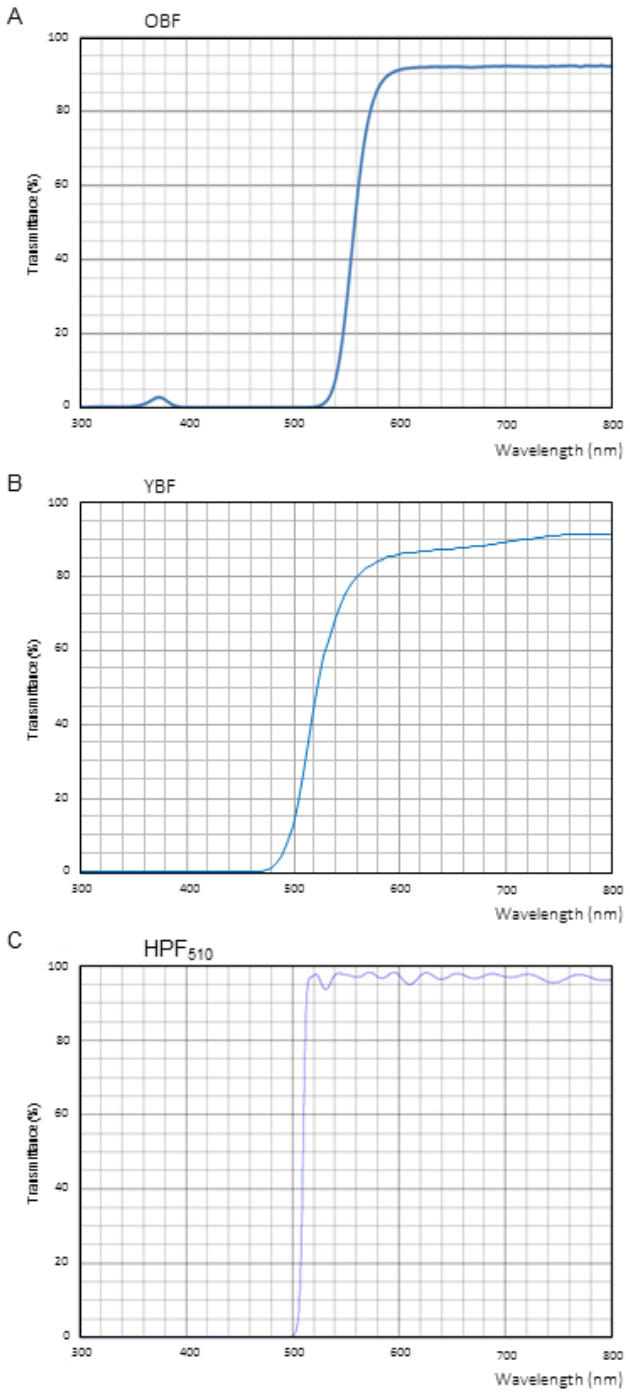

A
OBF
Transmittance (%)
Wavelength (nm)
B
YBF
Transmittance (%)
Wavelength (nm)
C
HPF510
Transmittance (%)
Wavelength (nm)

Fig. S3

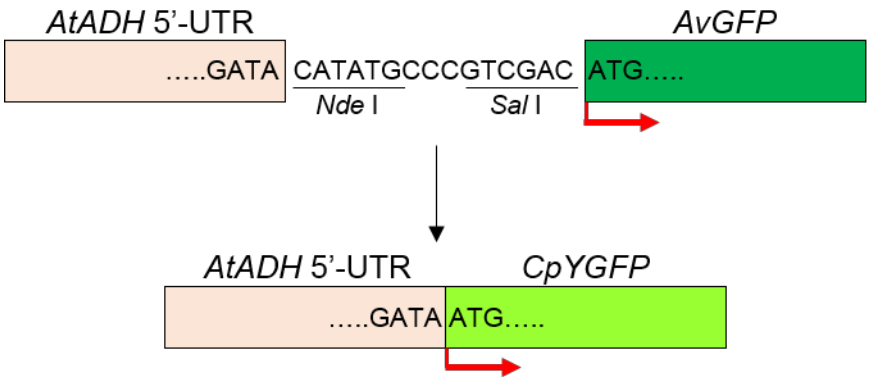

AtADH 5'-UTR
AvGFP
.....GATA
CATATGCCCGTCGAC
ATG.....
Nde I
Sal I
AtADH 5'-UTR
CpYGFP
.....GATA
ATG.....

Fig. S4

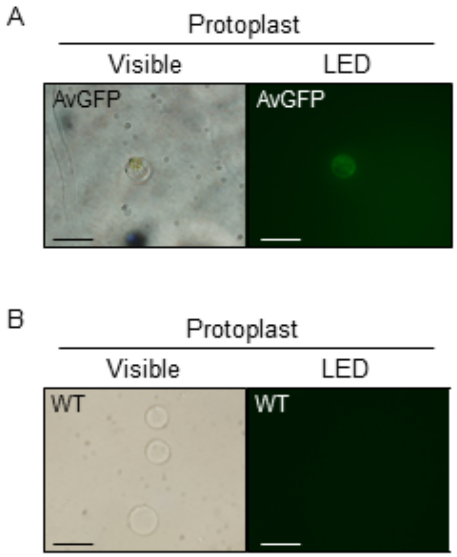

A
Protoplast
Visible
LED
AvGFP
AvGFP
B
Protoplast
Visible
LED
WT
WT

Fig. S5

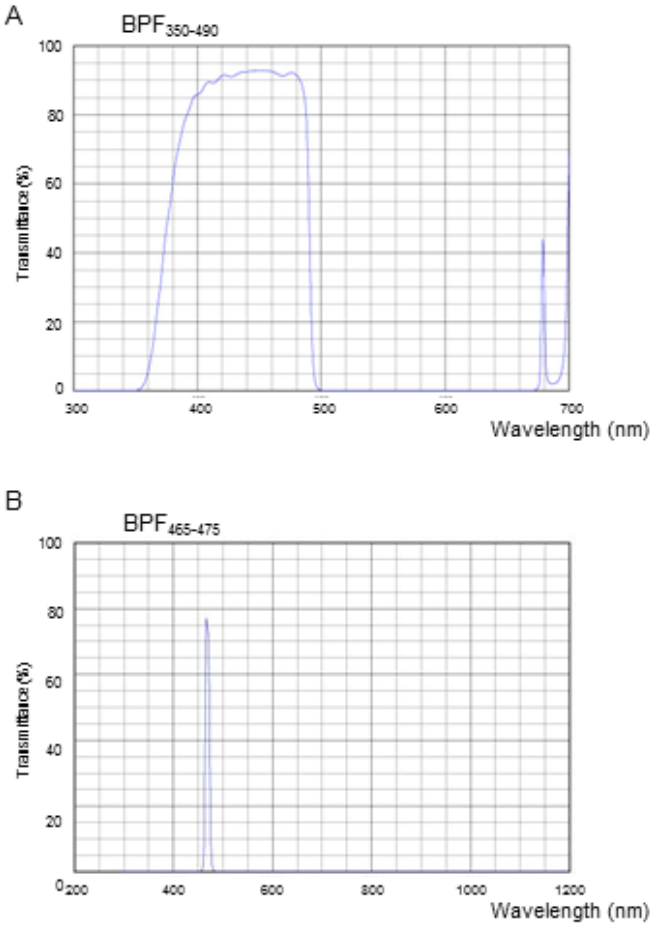
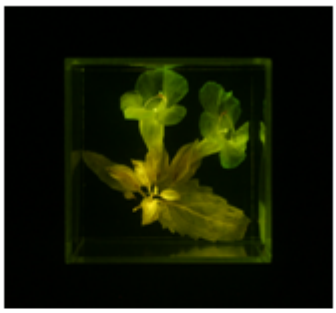

A
BPF350-490
100
80
60
40
20
0
Transmittance (%)
300
400
500
600
700
Wavelength (nm)
B
BPF465-475
100
80
60
40
20
0
Transmittance (%)
200
400
600
800
1000
1200
Wavelength (nm)

Fig. S6

A

LED_{459} / YBF / $BPF_{350-490}$



B

Fig. S7

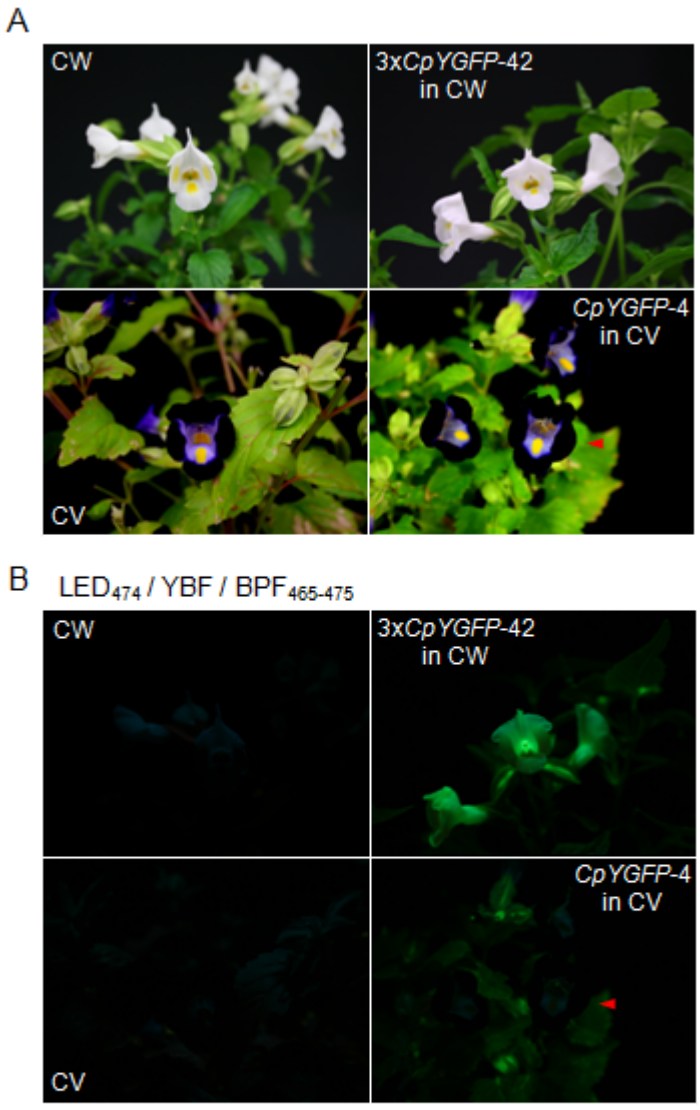

A
CW
3x*CpYGFP*-42
in CW
CV
CpYGFP-4
in CV
B
LED474 / YBF / BPF465-475
CW
3x*CpYGFP*-42
in CW
CV
CpYGFP-4
in CV
C
WT 3 4 5 6 7 13 14 17 20
CpYGFP
TfACT3
24
25

Fig. S8


A
WT
3xCpYGFP
B
LED459 / HPF510 / BPF465-475
WT
3xCpYGFP
oblique

Fig. S9